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Salvianolic acid A suppresses MMP-2 expression and restrains cancer cell invasion through ERK signaling in human nasopharyngeal carcinoma

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ABSTRACT

Ethnopharmacological relevance: Salvia miltiorrhiza Bunge, as known as Danshen, has used for the prevention and treatment of cardiovascular diseases clinically and anti-cancer activities. Salvianolic acid A (SAA), one of the most abundant ingredients, hydrophilic derivatives of *Salvia miltiorrhiza* Bunge, exerts a variety of pharmacological actions, such as anti-oxidative, anti-inflammatory and anti-cancer activities. However, the impact of SAA on nasopharyngeal carcinoma (NPC) invasion and metastasis remains unexplored.

Aim of the study: To investigate the potential of SAA to prevent migration and invasion on NPC cell.

Materials and methods: MTT assay and Boyden chamber assay were performed to determine cell proliferation, migration and invasion abilities, respectively. The activity and protein expression of matrix metalloproteinase-2 (MMP-2) were determined by gelatin zymography and western blotting.

Results: Here, we showed that SAA considerably suppressed the migrative and invasive activity of human NPC cells but not rendered cytotoxicity. In SAA-treated NPC cells, the activity and expression of matrix metalloproteinase-2 (MMP-2), a key regulator of cancer cell invasion, were reduced. Additionally, the presence of high concentrations of SAA dramatically abolished the activation of focal adhesion kinase (FAK) and moderately inhibited the phosphorylation of Src and ERK in NPC cells.

Conclusions: Our results demonstrated that SAA inhibited the migration and invasion of NPC cells, accompanied by downregulation of MMP-2 and inactivation of FAK, Src, and ERK pathways. These findings indicate a usefulness of SAA on restraining NPC invasion and metastasis.

1. Introduction

Nasopharyngeal carcinoma (NPC), a malignancy that originates from the epithelial lining of the nasopharynx, is common in Southeast Asia, with an annual incidence of ~30 per 100,000 subjects (Wei and Sham, 2005). In addition to genetic susceptibility and infection with Epstein-Barr virus (EBV), the etiology of NPC involves a variety of environmental risk factors associated with the unique cultural practice and life style of different ethnic populations (for example, smoking and consumption of preserved food) (Chua et al., 2016). These etiological parameters across distinct geographical regions in part account for the heterogeneity of global incidence, histologic types, and treatment

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response for this disease. Currently, the treatment of NPC has improved significantly, with radiotherapy alone (for early-stage disease) or combined chemo-radiotherapy (for locally advanced tumors) being the mainstay of therapy for NPC. However, treatment failure, largely in the form of local recurrence or regional/distant metastases, is still present in approximately 20% of patients following primary therapy (Au et al., 2018; Cao et al., 2013). Recurrent and metastatic NPC are often refractory to advanced therapy and associated with high mortality (Razak et al., 2010). Therefore, a better understanding of the molecular mechanisms underlying NPC metastasis is essential for providing insights into the development of novel therapeutic approaches, ultimately ameliorating the prognosis.

Tumor metastasis, the major cause of deaths due to malignancies, is a multistage process that requires cancer cells to escape from the primary location, survive in the circulation, and grow at distant sites (Coghlin and Murray, 2010; Hsiao et al., 2019; Su et al., 2017). Metastasis of NPC is regulated by diverse cellular and organismic mechanisms rendered by both cancer and non-neoplastic cells within the tumor microenvironment (Chambers et al., 2002; Joyce and Pollard, 2009; Reymond et al., 2013). These include activation of signaling pathways, regulation of cell-cell and cell-matrix interaction, extracellular matrix (ECM) remodeling by matrix metalloproteinases (MMPs), cytoskeleton reorganization, elevated mobility, escape from cell apoptosis, epithelial-mesenchymal transition (EMT), and angiogenesis (Chien et al., 2013; Lee et al., 2019a, 2019b; Su et al., 2017).

In Traditional Chinese Medicine (TCM), *Salvia miltiorrhiza* Bunge (Danshen), belonging to the Labiatae family of flowering plants, is a widely used for the prevention and treatment of cardiovascular and endocrine diseases clinically (Wang et al., 2018) and anti-cancer activities (Chen et al., 2014). Moreover, Lin et al., also validated the in vivo protective effect of *Salvia miltiorrhiza* Bunge in the different stage of colon cancer patients (Lin et al., 2017). A number of active ingredients for *S. miltiorrhiza*, either water-soluble or lipid-soluble, has been isolated, including salvianolic acid A (SAA), salvianolic acid B and salvianolic acid C and so on (Liu et al., 2007). Among them, salvianolic acid A, one of the major compound of *S. miltiorrhiza*, exerts a variety of pharmacological actions, such as anti-oxidative (Zhang et al., 2014a), anti-inflammatory (Oh et al., 2011), anti-diabetic (Qiang et al., 2015), and anti-thrombotic effects (Fan et al., 2010). The potential effect of SAA for cancer treatment has been proposed (Ma et al., 2019). Specifically, SAA resensitized breast cancer cells to chemotherapy via targeting the expression of an actin-binding protein, transgelin 2 (Cai et al., 2014; Zheng et al., 2015). In addition, SAA was shown to suppress cancer cell proliferation by acting as an inhibitor of the endothelin-1 receptor (Zhang et al., 2016). These findings point out a functional role of SAA in promoting cancer cell apoptosis. Yet, the impact of SAA on NPC metastasis remains poorly understood. Therefore, the aim of the present study is to test the activity of SAA on affecting the migration and invasiveness of NPC cells and to explore the molecular mechanisms associated with SAA-regulated NPC cell motility.

2. Materials and methods

2.1. NPC cell culture and reagents

Human NPC cell line, HONE-1, was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). NPC-39, established from a patient with NPC (Liao et al., 1998), was given by Dr. MK Chen, Department of Otolaryngology, Changhua Christian Hospital, Changhua, Taiwan. Cells were propagated in RPMI-1640 medium containing 10% FBS and maintained at 37 ˚C in a humidified atmosphere of 5% CO2. Salvianolic acid A (SAA), of HPLC grade with ≥98% purity, was obtained from Sigma-Aldrich (St. Louis, MO, USA) and prepared in sterile distilled water.

2.2. Measurement of cell viability

The cytotoxicity of SAA is determined by assessing cell viability using a microculture tetrazolium (MTT) colorimetric assay as previously described (Lu et al., 2018). Different concentrations of SAA were added into the 24-well plates and incubated for 24 h. The measurement of visible cells was based on the production of formazan following solubilization with isopropanol, which was estimated spectrophotometrically at 563 nm in a spectrophotometer (DU640, Beckman Instruments, Fullerton, CA).

2.3. Wound healing assay

8×10^5 cells were seeded in 6-cm plates for 24 h. Prior to creating a scratch with a yellow, cells were serum starved overnight. After cell debris was removed by washing with PBS, cells were maintained in a condition medium containing 0.5% FBS and indicated concentrations of SAA. Cell culture was photographed at 0, 12, and 24 h by using an Olympus CKX41 phase contrast microscope (Olympus Corporation, Tokyo, Japan) at 100 × magnification to determine the width of the remaining wound area relative to the initial wound width.

2.4. Cell migration and invasion assay

We employed a modified Boyden chamber assay without and with 10 μL of Matrigel (25 mg/50 mL; BD Biosciences, MA) coating to assess cell migration and invasion, respectively, as described previously (Lu et al., 2018). In brief, cells were pretreated with indicated concentrations of SAA for 24 h and then seeded on the 8-μm-pore size polycarbonate membrane filter at 10^4 cells/well in serum-free media and the bottom chamber contained a standard culture medium (10 % fetal bovine serum). Cells were allowed to migrate or invade for 24 h and counted under an Olympus CKX41 microscope (Olympus Corporation, Tokyo, Japan).

2.5. Gelatin zymography

The gelatinolytic activities of matrix metalloproteinase-2 (MMP-2) in culture medium were measured by using gelatin zymography protease assays as described previously (Lin et al., 2014). Conditioned media of an appropriate volume were subjected to 0.1% gelatin (Sigma-Aldrich, St. Louis, MO) and 8% SDS-PAGE. After gel electrophoresis, gels were washed with 2.5% Triton X-100, incubated in reaction buffer (40 mM Tris-HCl, pH 8.0; 10 mM $CaCl_2$ and 0.01% (w/v) NaN_3) for 24 h at 37 ˚C, and then stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich, St. Louis, MO, USA).

2.6. Immunoblotting

Total protein lysates (20 μg) of cell cultures were subjected to SDS-PAGE and transferred to Immobilon PVDF membranes (Millipore, Bedford, Massachusetts, USA). Antibodies against the following proteins were used for detection: Anti-p38a (1:1,000 dilution) and anti-β-actin Ab5 (1:5,000 dilution) from BD Biosciences (Bedford, MA, USA); Anti-MMP-2 (1:1,000 dilution), Anti-phospho-FAK (Tyr925) (1:1,000 dilution), Anti-FAK (1:1,000 dilution), Anti-phospho-Src (1:1,000 dilution), Anti-Src (1:1,000 dilution), Anti-phospho-p38 (Thr180/Tyr182) (1:1,000 dilution), anti-p44/42 (ERK1/2) (1:1,000 dilution), anti-phospho-p44/42 (ERK1/2) (1:1,000 dilution), anti-SAPK/JNK (1:1,000 dilution), anti-phospho-SAPK/JNK (Thr183/Tyr185) (1:1,000 dilution) antibodies from Cell Signaling Technology (Danvers, MA, USA); HRP-conjugated secondary antibodies (1:5,000 dilution) (Dako Corporation, Carpinteria, CA, USA). Densitometric analysis of blots was performed using ImageJ software.

2.7. Quantitative PCR

Total RNA was extracted using an RNeasy Mini Kit according to the manufacturer's instructions (QIAGEN, Valencia, CA, USA). Each real-time PCR reaction contained 0.5 ng/μL of cDNA and 400 nM of each primer in a 25-μL reaction volume. The reaction was initiated at 94 °C for 1.5 min, followed by 40 two-step amplification cycles consisting of 15 s of denaturation at 95 °C and 45 s of annealing/elongation at 60 °C in the Applied Biosystems StepOne Real-time polymerase chain reaction (PCR) system (Applied Biosystems, Foster City, CA, USA). Assays were performed in triplicate against three independent preparations of cDNA. For each reaction, a threshold cycle was observed in the exponential phase of amplification, and the quantification of relative expression levels was achieved using standard curves for both MMP-2 (Hs00234422_m1; FAM-GCAGGGCGGCGGTCACAGCTACTTC) and a constitutively expressed gene, GAPDH (Hs99999905_m1; FAM-GGCG CCTGGTCACCAGGGCTGCTTT), whose expression changed < 1.15-fold.

2.8. Statistical analysis

Data were shown as means ± standard deviation (SD) of at least three independent experiments. A P value of < 0.05 was considered statistically significant by using Student's t-test.

3. Results

3.1. Effect of SAA on NPC cell viability

Suppressive effects of SAA on cell proliferation have been detected in multiple cancer cell lines (Tang et al., 2017; Zhang et al., 2016; Zheng et al., 2015). To determine whether SAA is cytotoxic to NPC cells, NPC-39 and HONE-1 cells were treated with different concentrations of SAA, ranging from 0 to 50 μM, and examined for cell viability. Unlike the anti-proliferative responses observed in other cancer cells, SAA, even at 50 μM, did not alter cell viability of NPC cells (Fig. 1).

3.2. Effect of SAA on NPC cell motility

Next, the impact of SAA on NPC cell mobility was tested by performing an in vitro wound healing assay and a modified Boyden chamber assay. We found that high concentrations of SAA (at 25 and 50 μM) rendered suppressive effects on cell motility at 12 h post-treatment in both HONE-1 and NPC-39 cells without altering the cell proliferation (Fig. 2A & B). Moreover, at 24 h post-treatment, SAA consistently inhibited the motility, migration, and invasion of both HONE-1 and NPC-39 cells in a dose-dependent manner (Fig. 2C–D). These results reveal a usefulness of SAA on restricting NPC invasion and metastasis.

3.3. SAA suppresses the activity and expression of MMP-2 in NPC

Since matrix metalloproteinase-2 (MMP-2) is an important determinant of cancer cell invasion through remodeling extracellular matrices (Xu et al., 2005), the potential involvement of MMP-2 in SAA-mediated inhibition of NPC invasion was then investigated. To address this, conditioned media of SAA-treated NPC cells were collected and applied to a gelatin zymography assay for assessing the activities of MMP-2. We noted a significant decrease in gelatin digestion upon SAA treatment in the culture media of both HONE-1 and NPC-39 cells (Fig. 3A), indicating that SAA efficiently suppresses the activities and extracellular levels of MMP-2 in NPC. Further, the intracellular levels of MMP-2 in SAA-treated NPC cells were examined at the protein and RNA level. We observed that SAA consistently downregulated the protein (Fig. 3B) and RNA (Fig. 3C) of MMP-2 in both cell lines tested. These results suggest that SAA is capable of reducing the activity and expression of a key player of cell invasion, MMP-2, in NPC.

3.4. SAA inhibits FAK, Src, and ERK pathways in NPC

Diverse intracellular signaling pathways, such as integrin (Yue et al., 2012) and MAPK (Boyd et al., 2005; Yang et al., 2019; Yeh et al., 2019) signaling, are known to regulate MMP-2 expression and play a vital role in cancer invasion and metastasis. We, next, tested whether SAA mediates the activation of focal adhesion kinase (FAK), Src, ERK, JNK, and p38 in NPC cells. We found that high concentrations of SAA almost abolished the activation of FAK and moderately inhibited the phosphorylation of Src in both HONE-1 and NPC-39 cells (Fig. 4A). In term of MAPK, SAA, at high concentrations, decreased the activation of ERK, whereas it did not affect that of JNK and p38 (Fig. 4B). To further investigate whether the suppression of the cell migration by SAA was mainly caused by the inhibition of the MEK/ERK1/2 signaling pathway, the MEK inhibitor (U0126) and ERK1/2 activator (t-BHQ) were used to confirm the mechanism in NPC-39 cells. As shown in Fig. 5A, the cell migratory ability was suppressed when the cells were treated with SAA only. The combination treatment of U0126 (5 μM) and SAA (25 μM) resulted in the intensive inhibition of the migratory ability in NPC-39 cells. Otherwise, the ERK1/2 activator (t-BHQ) significantly increased the cell migratory abilities of NPC-39 cells and also reversed the SAA-mediated suppression of the migratory ability in NPC-39 cells (Fig. 5B).

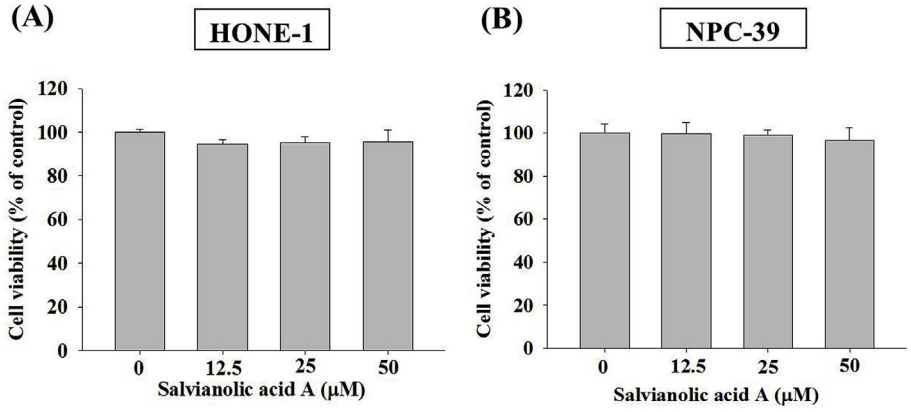

Fig. 1. SAA is not cytotoxic to human NPC cells. HONE-1 and NPC-39 cells were treated with various concentrations (0–50 μM) of SAA for 24 h and then examined for cell viability. The values represented the means ± SD from at least three independent experiments.

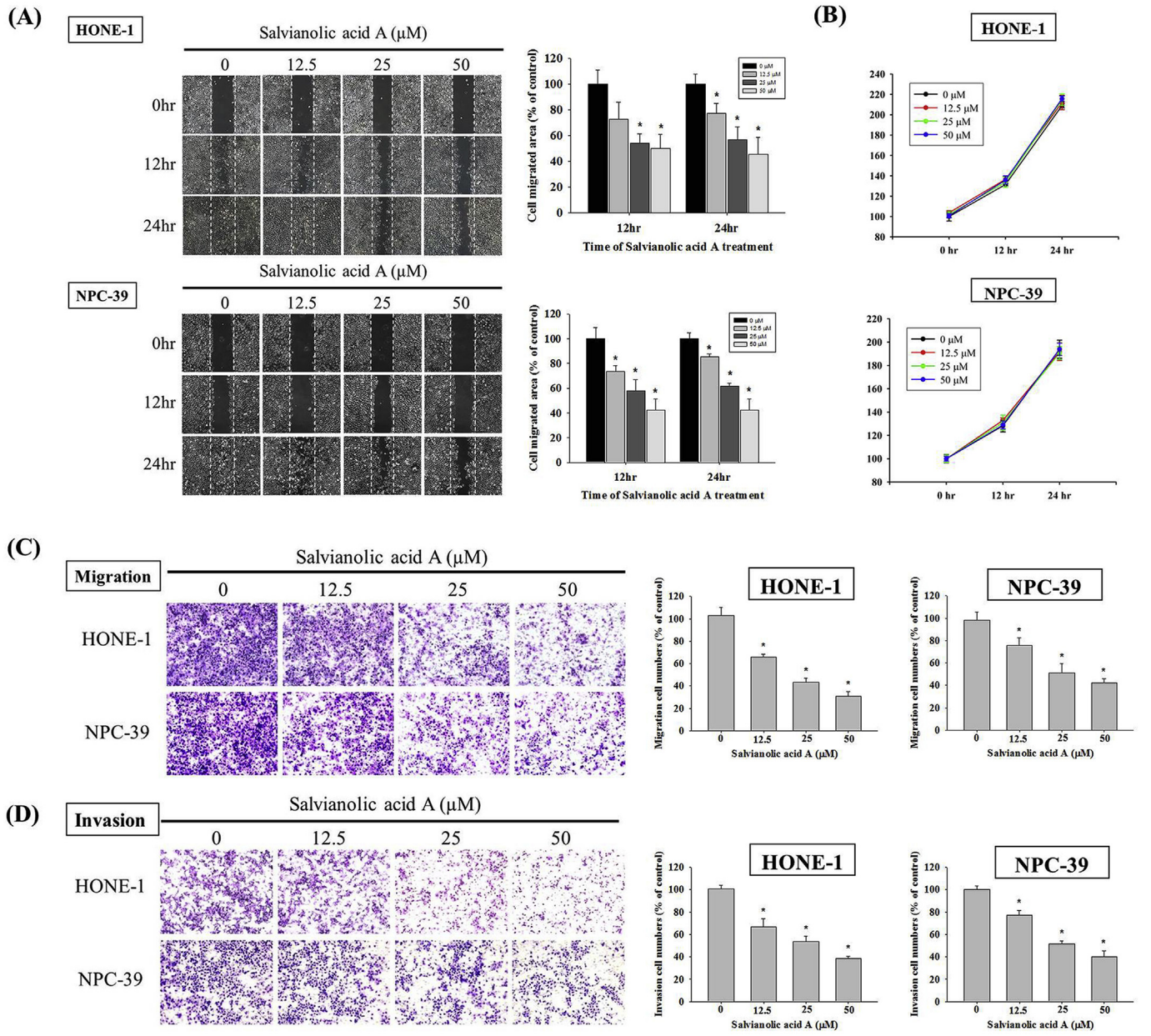

Fig. 2. SAA suppresses the motility, migration, and invasion of NPC cells. (A) Confluent monolayers of HONE-1 and NPC-39 cells were scratched and treated with indicated concentrations of SAA. Wound closure was monitored at 12 and 24 h post-treatment. The right panel is quantification of cell motility. **(B)** HONE-1 and NPC-39 cells were treated with various concentrations (0–50 μM) of SAA for 12 and 24 h and then examined for cell proliferation. **(C–D)** HONE-1 and NPC-39 cells were pretreated with indicated concentrations of SAA for 24 h. Cell migration **(C)** and invasion **(D)** were assayed at 24 h after seeding in a modified Boyden chamber without and with Matrigel coating, respectively. Quantitative data are shown in the right panel. *, $p < 0.05$ as compared with SAA-untreated controls.

4. Discussion

Although current standard management of NPC has obtained favorable outcomes in patients with early-stage diseases, metastases remain a huge challenge for the treatment of NPC. Thus, alternative therapeutic approaches are needed to improve patients' survival and quality of life. A large body of evidence has shown that medicinal herbs and their derivatives are beneficial for combating malignancies, when used in combination with conventional therapeutics (Yin et al., 2013). Here, we demonstrated that SAA, one of the most abundant water-soluble derivatives of *S. miltiorrhiza*, exerted suppressive effects on NPC invasion and migration. Further characterization of the underlying molecular mechanisms revealed that the inhibition of NPC motility by SAA was accompanied by reducing MMP-2 activity and expression and hampering activation of FAK, Src, and ERK signaling pathways. Our findings, for the first time, indicate a usefulness of SAA on restraining NPC invasion and metastasis.

Numerous studies have proposed diverse anti-cancer effects of SAA on treating various types of cancer (Ma et al., 2019). In multidrug-resistant breast cancer cells, SAA promoted programmed cell death by increasing the activity of caspase-3 (Wang et al., 2015) and inhibited the invasion responses by modulating the expression of many junctional proteins, such as E-cadherin and N-cadherin (Zheng et al., 2015). Here, we found that SAA considerably suppressed the migrative and invasive activity of human NPC cells but exhibited no effect on cell apoptosis. Notably, we observed that the inhibition of NPC invasion by SAA was accompanied by inactivation of Src and FAK (Fig. 4A). Mounting evidence from in vitro and in vivo experiments has placed these two nonreceptor protein tyrosine kinases at the heart of E-cadherin regulation and the crosstalk between integrins and cadherins (Serrels et al.,

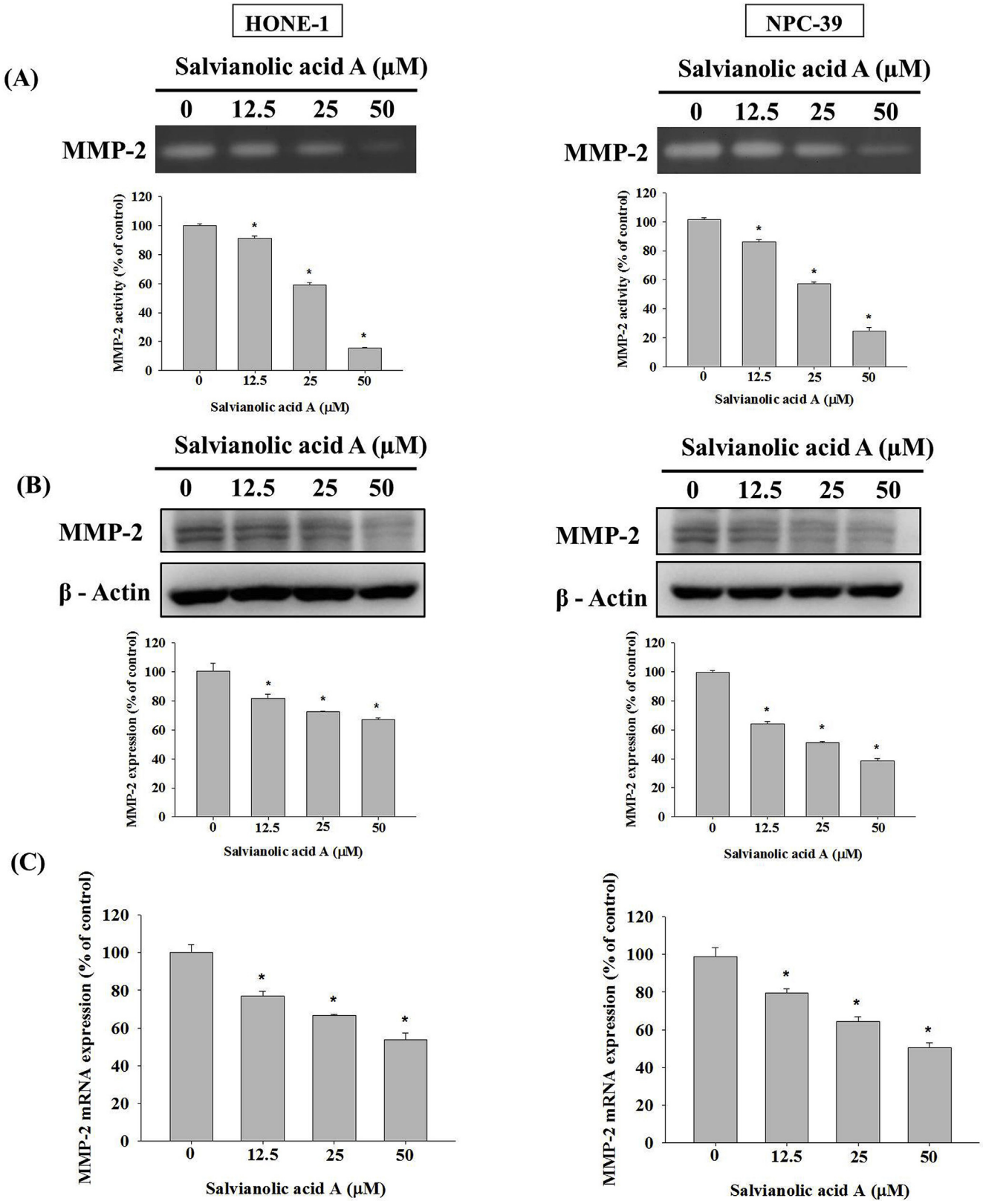

Fig. 3. SAA inhibits the activity and expression of MMP-2 in NPC cells. HONE-1 and NPC-39 cells were treated with SAA (0–50 μM) for 24 h. Conditioned media were subjected to gelatin zymography for analyzing the activity of MMP-2 **(A)**. Cell lysates and total RNA were prepared for determining the levels of MMP-2 protein **(B)** and RNA **(C)**. Densitometric analyses of gelatin zymography and immunoblots were perfomed by the ImageJ software. The expression of MMP-2 protein and RNA were normalized to the levels of β-actin protein and GAPDH RNA, respectively. *, $p < 0.05$, compared with the untreated control.

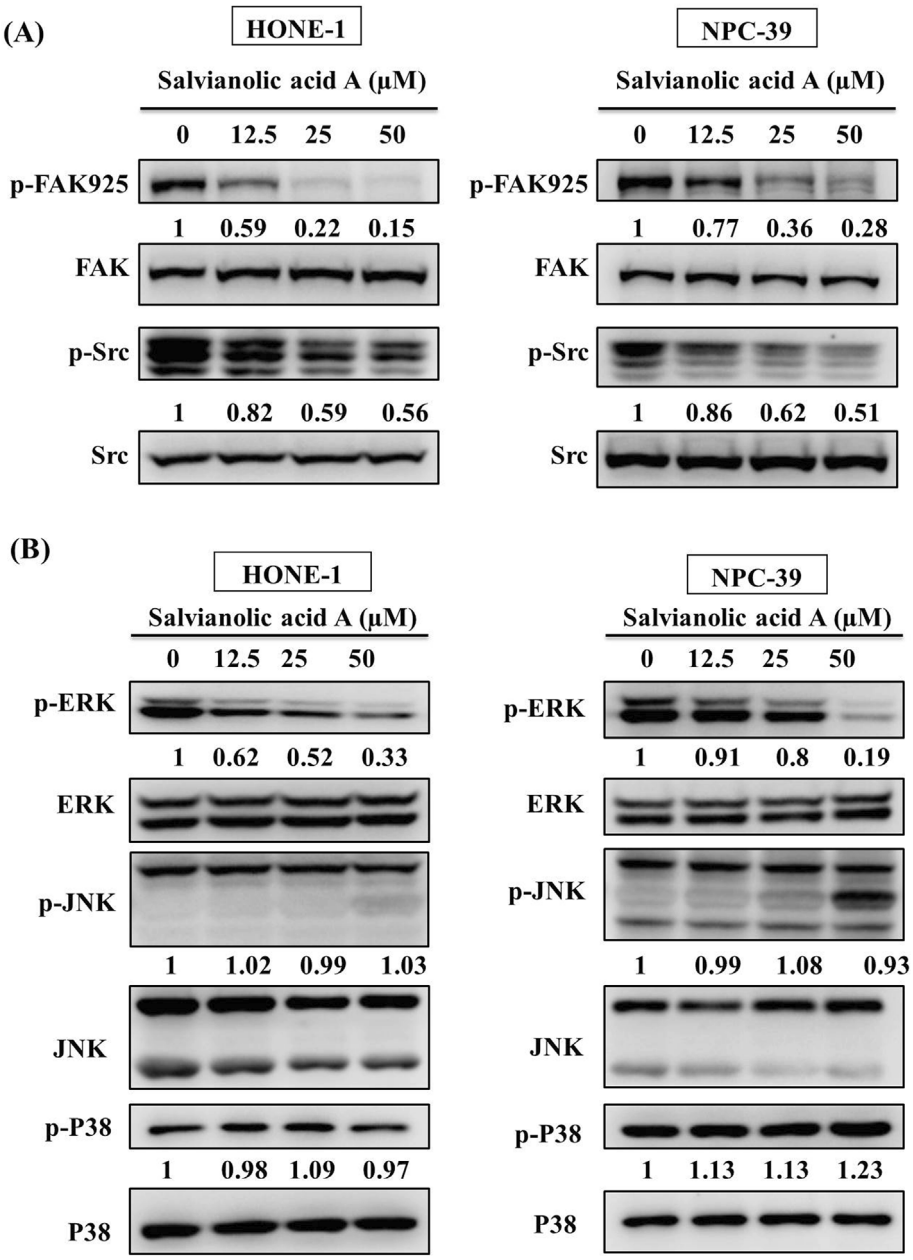

Fig. 4. Effect of SAA on regulating the adhesion (A) and MAPK (B) signaling pathways. HONE-1 and NPC-39 cells were treated with SAA (0–50 μM) for 24 h, and cell lysates were subjected to Western blot analysis to analyze the phosphorylation of FAK and Src for adhesion signaling (A), as well as ERK, JNK, and p38 for MAPK pathways (B). Densitometric analyses of kinase phosphorylation were conducted by ImageJ. Blots are representative of three independent experiments.

2011). These findings indicate a potential involvement of a regulatory crosstalk between the Src/FAK signaling axis and cadherins in SAA-mediated inhibition of NPC invasion. In addition to adhesion signaling, treatment of SAA in cancer was shown to orchestrate different MAPK pathways. Unlike interrupting JNK pathway in lung cancer (Li et al., 2002), we noted that SAA decreased activation of ERK but not that of p38 and JNK pathway in NPC (Fig. 4B). Furthermore, SAA has been demonstrated to decrease cell migration in vivo by modulating MMP-9 (Jiang et al., 2013; Zhang et al., 2014b). In the present study, we showed that SAA suppressed the activity and expression of another gelatinase, MMP-2. Consistently, down-regulation of both MMP-9 and MMP-2 in oral cancer by salvianolic acids have been reported (Fang et al., 2018; Yang et al., 2011). The results from our and others' investigations implicate the use of salvianolic acids as a promising modality against the angiogenic and metastatic potential of head and neck cancers via targeting MMP-2/9.

Our results reveal a suppressive effect of SAA on NPC invasion; nevertheless, extra work is needed to address several limitations of this study. First, even though we showed the inhibition of NPC invasion by SAA in the culture of human NPC cell lines, the capacity of this salvianolic acid may be altered after ingestion and absorption in the human body. Additional in vivo experiments are required to verify its clinical value for NPC treatment. Second, both cell lines used in this study are Epstein-Barr virus (EBV)-negative, yet the major histologic types of NPC (nonkeratinizing carcinoma and undifferentiated carcinoma) are predominantly EBV-positive (Wei and Sham, 2005). Acquisition of EBV-positive NPC cell lines for testing the impact of SAA will improve the clinical relevance.

Taken together, our data for the first time demonstrated that SAA suppressed the migration and invasion of NPC cells, accompanied by downregulation of MMP-2 and inactivation of FAK, Src, and ERK pathways (Fig. 5C). These findings provide novel insights into the use of

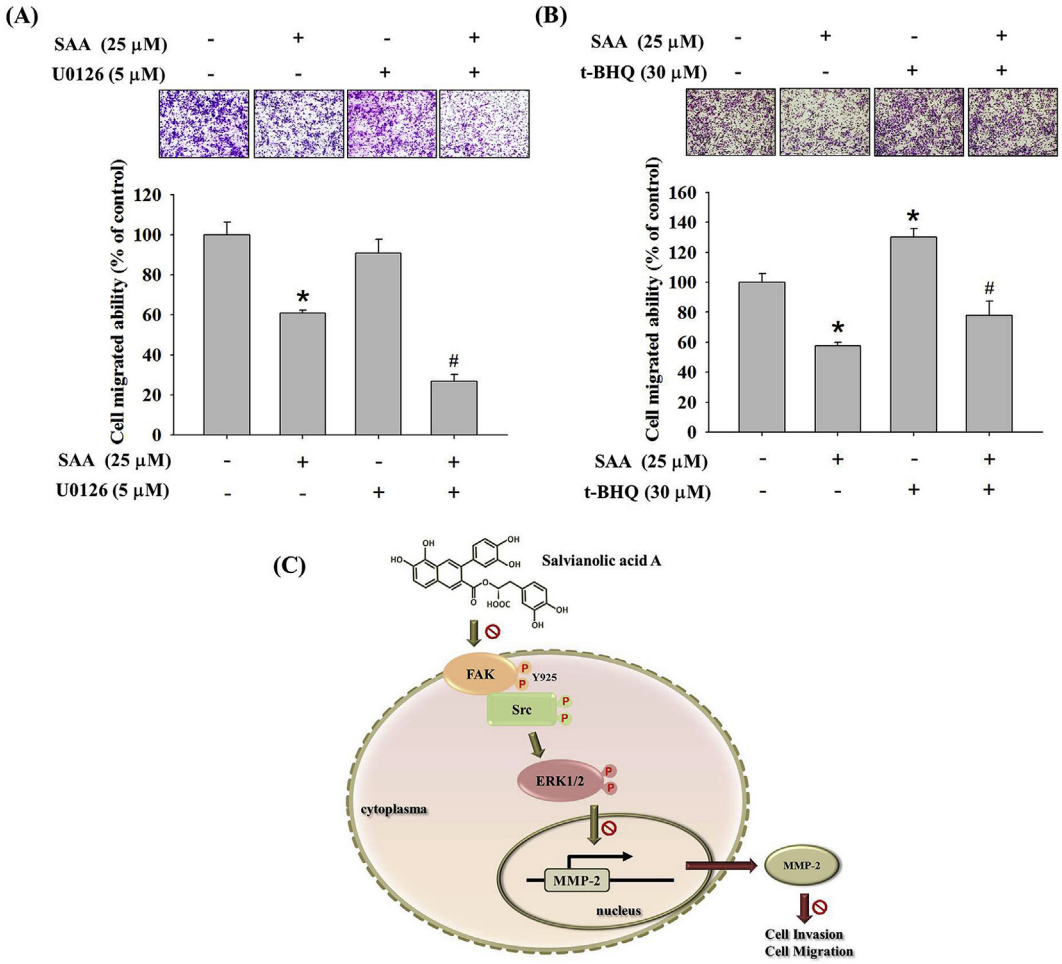

Fig. 5. Inhibitory effect of MEK inhibitor (U0126) and ERK1/2 activator (t-BHQ) on cell migration. (A) Human NPC-39 cell lines were pre-treated with U0126 (5 μM) for 1 h and then incubated in the presence or absence of SAA (25 μM) for 24 h and the migration ability of cells were observed by Boyden chamber assay. (B) Human NPC-39 cell lines were pre-treated with SAA (25 μM) for 1 h and then incubated in the presence or absence of t-BHQ (30 μM) for 24 h. The migration ability of cells was observed by Boyden chamber migration assay. *$p < 0.05$, compared with the vehicle group. $^{\#}p < 0.05$, compared with the U0126 or t-BHQ treated group. (C). Schematic diagram for proposed signaling pathways in the inhibitory mechanisms of SAA on cell migration in human nasopharyngeal carcinoma cells. SAA suppresses the level of phosphorylate-FAK, Src, ERK signaling pathway, and furthermore inhibits MMP-2, leading to reduced nasopharyngeal carcinoma cell motility, invasion and migration.

SAA on restraining NPC invasion and metastasis.

Author contributions

CY Chuang, YC Ho, SF Yang and SC Su designed and conceived the study; WE Yang, CW Lin, MC Tsai and SF Yang performed the experiments. YL Yu and SF Yang performed the statistical analysis. CY Chuang, YC Ho, SF Yang and SC Su drafted the article and made critical revisions to the manuscript.

Declaration of competing interest

The authors have declared there is no competing interest exists.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2020.112601.

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